

# Laboratory Bioassays to Evaluate Fungicides for Chalkbrood Control in Larvae of the Alfalfa Leafcutting Bee (Hymenoptera: Megachilidae)

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**ABSTRACT** Chalkbrood, a fungal disease in bees, is caused by several species of *Ascosphaera*. *A. aggregata* is a major mortality factor in populations of the alfalfa leafcutting bee, *Megachile rotundata* (F.) (Hymenoptera: Megachilidae) used in commercial alfalfa seed production. Four formulated fungicides, Benlate 50 WP, Captan, Orbit, and Rovral 50 WP were tested in the laboratory for efficacy against hyphal growth of *A. aggregata* cultures. The same fungicides, with the addition of Rovral 4 F, were tested for their effects on incidence of chalkbrood disease, and toxicity to *M. rotundata* larvae. Benlate, Rovral 50 WP, and Rovral 4 F reduced incidence of chalkbrood with minimal mortality on larval bees. Benlate and Rovral 50 WP also reduced hyphal growth. Orbit was effective in reducing hyphal growth, but it did not reduce incidence of chalkbrood and was toxic to bee larvae. Captan was not effective in reducing hyphal growth or chalkbrood incidence, and it was toxic to bee larvae. Fungicides that reduce incidence of chalkbrood and larval mortality in this laboratory study are candidates for further study for chalkbrood control.

**KEY WORDS** alfalfa leafcutting bee, bees, chalkbrood, disease control, insect pathology

Pollination of alfalfa, *Medicago sativa* L., seed crops in the United States and Canada primarily rely on managed populations of the alfalfa leafcutting bee, *Megachile rotundata* (F.) (Hymenoptera: Megachilidae). Populations of this solitary bee are released in nesting shelters provided with wood or polystyrene boards as nesting substrates (Frank 2003). Female *M. rotundata* use the holes drilled in these boards to build multicelled nests lined with leaf cuttings. Each completed cell contains a pollen–nectar provision and a single egg. Upon consuming the pollen–nectar provision, the bee larva spins a cocoon and enters diapause (Kemp et al. 2004). Pupation and adult emergence occur the following summer. A major problem associated with managing *M. rotundata* on a commercial scale is chalkbrood. Chalkbrood is a fungal disease infecting bee larvae caused by species of *Ascosphaera*. The fungus infects larvae that have consumed pollen and nectar contaminated with its spores. *Ascosphaera aggregata* Skou, is the primary source of chalkbrood in commercially managed populations of *M. rotundata*. The spores germinate in the bee's gut, spreading hyphae throughout the host, ultimately killing the bee (Vandenberg and Stephen 1982, McManus and

Youssef 1984). *M. rotundata* mortality from chalkbrood averages 10 to 35% in the northwestern United States (James and Huntzinger 2003), and infection levels in excess of 50% are not uncommon (Undurraga 1978, Peterson et al. 1992). Strategies to control chalkbrood have focused on reducing spore contamination on nesting boards and equipment used in managing the bees, all with inadequate results (Frank 2003; James 2005; Parker 1984, 1985, 1987, 1988). As a consequence, alfalfa seed growers in the United States must supplement their bee populations annually by purchasing bees from areas of Canada that have very low chalkbrood levels.

Several studies have incorporated fungicides into pollen provisions to try to control chalkbrood in laboratory-reared larvae (Youssef and McManus 1985, Fichter and Stephen 1987, Youssef and Brindley 1989, Goettel et al. 1991, Vandenberg 1992). In vitro tests of this nature are useful not only to examine a fungicide's potential effectiveness in reducing chalkbrood incidence but also to estimate toxicity of a fungicide to *M. rotundata* larvae. Information on a fungicide's impact on chalkbrood disease and its toxicity to eggs and larvae will facilitate selection of potential fungicides for further, more elaborate studies targeted to chalkbrood control.

Measurements of survival of radial growth of fungal cultures have been used to evaluate fungicides and determine nutritional or environmental tolerance of the fungus to factors such as temperature and pH (Goettel et al. 1992, Everett and Neilson 1996, Ouedraogo et al. 1997, James et al. 1998, James 2004, Cole et al. 2005). Radial growth studies with *A. aggregata*

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have been done by James (2004) to determine upper and lower temperature thresholds, and by Goettel et al. (1992) to evaluate efficacy of five fungicides (benomyl, iprodione, enilconazole, ascocidin, and  $\alpha$ -DL-difluoromethyl ornithine).

In this study, the formulated fungicides Benlate 50 WP, Captan, Orbit, and Rovral 50 WP were screened for their relative effects on *A. aggregata* growth. The same fungicides, and the addition of Rovral 4 F, also were incorporated into the natural pollen provisions of *M. rotundata* to assess their efficacy in reducing chalkbrood disease and their effects on larval mortality.

### Materials and Methods

***A. aggregata* Bioassays.** Four fungicides were incorporated at various concentrations into agar media (see below) used to grow cultures of *A. aggregata* to obtain 0 to 100% inhibition of fungal growth. The trade mark name, active ingredients, and manufacturers of the fungicides used in this study are as follows: Benlate 50 WP (50% benomyl, EI DuPont, Wilmington, DE), Captan (48.9% captan, Micro Flo Company, Memphis, TN), Orbit (41.8% propiconazole, Novartis Crop Protection Inc., Greensboro, NC), and Rovral 50 WP (50% iprodione, Rhone-Poulenc Ag Company, Research Triangle Park, NC). Due to proprietary restrictions, the exact contents of the inactive ingredients were unavailable.

***A. aggregata* Cultures.** Cultures of *A. aggregata* came from a parent culture derived from a single spore isolated from a *M. rotundata* chalkbrood cadaver in 2002. Isolation of the spore was done by diluting the spore concentration in water and locating a single spore on an agar plate with a microscope. The spore and agar plug were transferred to a V-8 juice agar (James 2004). The isolate was designated Wild1 maintained at the USDA-ARS Pollinating Insects Research Unit in Logan, UT. Cultures were maintained on a V-8 juice agar as described by James (2004). Fungal cultures were prepared by transferring a plug from an established culture to a new petri plate and stored in an incubator set at 30°C, 80% RH in the dark for 7 d.

**Bioassay Method.** Fungicides were incorporated into media by suspending them in 1-ml sterile reverse osmosis (RO) water and then adding the suspension to 99 ml of autoclaved V-8 agar at 56°C before being poured into 100- by 15-mm petri plates. Control plates consisted of 99 ml of V-8 agar plus 1 ml of RO water free of any fungicides.

Circular plugs (5 mm in diameter) were cut from the margins of actively growing fungal cultures, and they were placed in the center of freshly made fungicide-agar plates. The plates were incubated in the dark at 30°C and 80% RH. After 7 d, radial growth was measured for each culture by averaging the diameter of the mycelial ring along two perpendicular lines that had been drawn previously on the cover of each plate, intersecting directly above the center of the plug.

**Experimental Design.** Different concentrations were tested in an attempt to obtain the range of

growth inhibition for each fungicide. For a given fungicide concentration, each replicate was set up on a different day and with a new preparation of the fungicide impregnated agar. Four plates were made each time a fungicide concentration was tested. Measurements from each cohort of four plates were averaged to make a single sample unit (replicate). The number of replicates and concentrations tested varied with each fungicide (Table 1). Regression analysis was used after a log transformation of fungicide concentration (the independent variable) by using Proc Reg (SAS Institute 1999) to determine the effect of fungicide concentration on *A. aggregata* growth rate (dependent variable) for each fungicide.

***M. rotundata* Egg Bioassays.** Fungicides were incorporated into the natural pollen provisions of *M. rotundata* eggs and raised in the laboratory to determine the effects on chalkbrood disease and bee mortality. In addition to the fungicides previously mentioned, Rovral 4 F (41.6% iprodione, Bayer Cropscience, KS City, MO) also was tested.

**Source of *M. rotundata* Eggs.** Eggs were from populations of *M. rotundata* in commercial alfalfa seed fields in Box Elder County, UT.

To obtain eggs, 18- by 40-cm polystyrene nesting blocks (Beaver Plastics Limited, Edmonton, AB, Canada) with paper straws (0.55 cm in diameter  $\times$  9.53 cm in length from Phoenix Tube Corp., Fort Wayne, IN) inserted into the holes were placed in shelters in a commercial alfalfa seed field that had high populations of nesting bees. The entire nesting block was periodically removed (about once a week) and immediately replaced by a new block. The nesting blocks were taken to the laboratory for extraction of the nest cells.

Nest cells were dissected from the straws, and the leaf caps were removed with forceps. Nest cells, with pollen provisions and eggs, were placed in plastic 96-well plates (well diameter 6.4 mm) and incubated overnight at 29°C. The next morning, eggs were examined under a dissecting microscope to eliminate eggs that had hatched, collapsed, or were parasitized. The remaining eggs were randomly assigned a treatment.

**Application of Fungicides.** Fungicides were mixed with RO water to a predetermined concentration, and 1  $\mu$ l of fungicide solution was injected below the surface of the pollen provision adjacent to the side of the egg (Goettel et al. 1991). This was done using a 50- $\mu$ l syringe attached to a Hamilton PB600-1 dispenser. Two controls were used. In the first, 1  $\mu$ l of sterile RO water was added to the provision. This water-treated control represented a fungicide concentration of zero. The second control had nothing added to the provision, and it was called the "untreated" control.

We tested the highest fungicide concentrations that did not clog the syringe (600, 400, 700, and 431.5 g [AI]/liter for Benlate, Rovral 50 WP, Captan, and Orbit, respectively). Each concentration (treatment) was replicated 10 times on different days with 7–10 bee eggs in each replicate.

Table 1. In the hyphal growth bioassay, several concentrations and replicates were tested for each fungicide; each replicate was set up on a different day

Fungicide Concن Tested (mg [AI]/liter)							
Benlate		Captan		Orbit		Rovral	
Concn	Replicates	Concn	Replicates	Concn	Replicates	Concn	Replicates
0.00	9	0.00	10	0.00	10	0.00	11
0.50	2	4.89	1	0.0086	1	0.50	3
1.00	4	9.78	3	0.0173	1	1.00	5
1.50	3	11.7	2	0.0259	1	1.50	1
2.00	3	12.2	2	0.0270	4	2.00	2
2.50	1	19.6	1	0.0280	1	3.00	1
3.00	2	24.5	2	0.0432	3	3.13	2
3.13	2	78.2	1	0.0863	2	3.75	1
3.50	1	97.8	3	0.1295	1	4.00	2
4.00	3	117	2	0.1726	2	5.00	1
4.50	1	313	1	0.2697	4	6.25	2
4.69	1	856	1	0.3452	2	7.50	1
5.00	2	978	3	0.3884	1	8.00	2
6.00	1	1,174	2	0.4315	1	9.00	1
6.25	4	1,223	3	0.6904	2	11.3	1
		1,252	1	0.7767	1	12.5	3
		1,712	1	0.8630	1	15.0	2
		2,445	3	1.1651	1	16.0	3
		3,423	1	1.2945	2	18.0	1
		5,007	1			20.0	4
		9,780	3			25.00	3
		19,560	1			27.00	1
		20,030	1			32.00	3
		29,340	1			54.00	2
		48,900	2				

To better define the dose-response curve of Orbit, dosages of 27, 53.9, 107.9, 215.8, and 431.5 g (AI)/liter were tested. These tests were replicated on four different days by using 16–55 bees per dosage. Rovral 4 F also was tested at dosages of 24.65, 49.3, 98.6, 197.2, and 394.4 g (AI)/liter. This dose-response test was repeated on three different days by using 24–173 larvae. The number of bees tested in each replicate varied due to the availability of eggs able to be collected.

Treatments were defined by the specific concentrations tested for each of the fungicides. Each replicate consisted of a cohort of eggs collected and treated on a different day from another replicate. Fungicide mixtures were made the day the replicate was treated.

**Rearing Conditions.** Once the bees were treated, the well plates were put into a 14.4-liter plastic container with a lid and placed in an incubator at 29°C. A 50-ml beaker of water was included in the container to increase humidity, and it was checked regularly and refilled as needed. When the larvae began to spin cocoons, lids were placed on the 96-well plates. Approximately 14–18 d after cocoon construction was complete, the bees were cooled to 22°C for 7 d, and then to 18°C for 2 wk, after which the larvae were put at 4°C for overwintering. The following spring (April), the cocoons were incubated at 29°C until the adult bees emerged from the cells.

The bees were evaluated just before cooling began for winter storage and later after they emerged as adults the following spring. The bees were classified into one of three categories: 1) healthy, those that cocooned, or emerged as adults; 2) chalkbrood, dead larvae with typical chalkbrood symptoms (see below);

and 3) other mortality, those larvae that died for some reason other than chalkbrood, including fungicide toxicity. In the spring, cocoons with unemerged bees were opened and the mortality was classified as either chalkbrood or other. Larvae classified as chalkbrood had either a typical graphite color from *A. aggregata* spores, or a light brown color from the cadaver being composed of hyphae.

**Data Analysis.** Mortality before winter storage of bees was not significantly different from adult emergence ( $P > 0.05$ ), so analysis was conducted on prewinter mortality. To normalize the distribution and variance of the proportions of bees categorized as healthy, chalkbrood, and other mortality, an arcsine-square root transformation was used. Tests in which only one fungicide concentration was tested used a Student's *t*-test to compare results with the water-treated control. Untreated controls also were compared with the water-treated controls with a Student's *t*-test. The tests that included several concentrations of Orbit and Rovral 4 F were analyzed using Dunnett's multiple comparisons test to compare mortality after exposure to each of the dosages with the water-treated controls.

**Results**

***A. aggregata* Bioassays.** After 7 d, *A. aggregata* growth in all the controls (treatments with no fungicides added) averaged 50.30 (SE = 1.03) mm. With sufficiently high doses, complete inhibition of hyphal growth was obtained for each of the fungicides when incorporated into the agar media. Orbit required the least amount of fungicide, ≈1.1 mg (AI)/L, to obtain

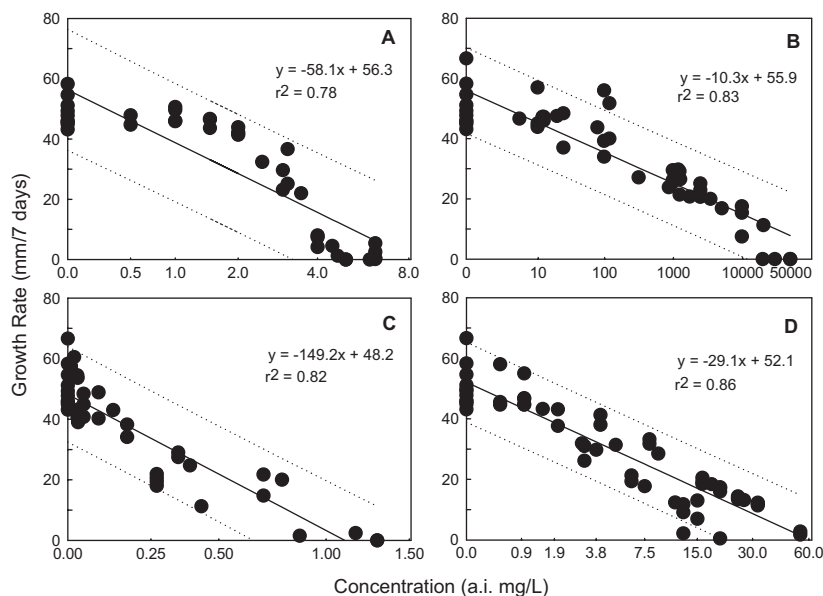


Fig. 1. Effect of fungicide concentrations on vegetative growth rates of *A. aggregata* cultures grown for 7 d with fungicides incorporated into the medium. Fungicides tested were (A) Benlate, (B) Captan, (C) Orbit, and (D) Rovral 50 WP. Dotted lines represent the upper and lower 95% confidence levels. The x-axes are different scales.

100% inhibition of hyphal growth (Fig. 1C). Captan required >30,000 mg (AI)/liter before complete inhibition of growth occurred (Fig. 1B). Benlate and Rovral 50 WP required  $\approx 6.0$  and 54.0 mg (AI)/liter, respectively, to obtain complete inhibition of vegetative growth (Fig. 1A and D).

The growth rate of *A. aggregata* was not affected by Benlate until concentrations exceeded 2.0 mg (AI)/liter, at which point vegetative growth was quickly inhibited (Fig. 1A). This lack of dose response at low rates resulted in a lower  $r^2$  value for Benlate ( $r^2 = 0.7847$ ) than the other fungicides. Adding an exponential term to the regression did not appreciably improve the fit.

***M. rotundata* Egg Bioassays.** In bioassays with eggs, the prevalence of chalkbrood, the number of healthy bees, and the number of bees killed by other mortality factors were not significantly different ( $P > 0.05$ ) between the water-treated controls and the untreated controls.

**Benlate.** The highest dose we were able to deliver with Benlate was 600 g (AI)/liter, which significantly increased ( $t = 3.87$ ,  $df = 9$ ,  $P = 0.0038$ ) the number of healthy bees, while lowering the incidence of chalkbrood ( $t = -5.43$ ,  $df = 9$ ,  $P = 0.0004$ ) (Fig. 2A). Other mortality also was significantly increased ( $t = 2.41$ ,  $df = 9$ ,  $P = 0.0394$ ).

**Captan.** The highest amount of Captan we were able to test was 700 g (AI)/liter. That level of Captan significantly decreased the percent of healthy bees ( $t = 5.35$ ,  $df = 9$ ,  $P = 0.0005$ ) (Fig. 2B). Other mortality increased significantly ( $t = 15.08$ ,  $df = 9$ ,  $P < 0.0005$ ), whereas chalkbrood levels were reduced ( $t = -4.03$ ,  $df = 9$ ,  $P = 0.0030$ ). Further intermediary concentrations were not tested because Huntzinger (2007)

found lower rates of Captan to be toxic to *M. rotundata* larvae, while failing to reduce chalkbrood levels.

**Orbit.** Orbit at 431.5 g (AI)/liter, increased other mortality significantly ( $t = 11.01$ ,  $df = 9$ ,  $P < 0.0001$ ) (Fig. 2C), and the percentage of healthy bees and chalkbrood incidence were reduced ( $t = -4.16$ ,  $df = 9$ ,  $P = 0.0025$  and  $t = -5.72$ ,  $df = 9$ ,  $P = 0.0003$ , respectively). When other concentrations were tested (Fig. 3), only the high concentration of 431.5 g (AI)/liter had any significant effects compared with the control. At the highest concentrations, chalkbrood was reduced ( $t = -3.09$ ,  $df = 15$ ,  $P = 0.0294$ ), but other mortality also increased ( $t = 4.37$ ,  $df = 15$ ,  $P = 0.002$ ); thus, the number of healthy bees was not significantly different from the control.

**Rovral.** Rovral 50 WP at 400 g (AI)/liter resulted in an increase in the percentage of healthy bees ( $t = 3.83$ ,  $df = 9$ ,  $P = 0.004$ ) (Fig. 2D). Chalkbrood was reduced ( $t = -7.07$ ,  $df = 9$ ,  $P < 0.0001$ ), and other mortality was increased ( $t = 2.95$ ,  $df = 9$ ,  $P = 0.0163$ ). The lowest concentration of Rovral 4 F tested, 24.65 g (AI)/liter, significantly increased the percentage of healthy bees ( $t = 4.70$ ,  $df = 10$ ,  $P = 0.0034$ ), reduced chalkbrood ( $t = -5.60$ ,  $df = 10$ ,  $P = 0.0009$ ), and it had no effect on other mortality (Fig. 4). Higher concentrations, up to 394.4 g (AI)/liter, also tended to increase the number of healthy bees and reduce chalkbrood. Concentrations higher than 24.65 g (AI)/liter increased other mortality, but not significantly.

The larval tests with a full dose response (Orbit and Rovral 4 F) both had higher mortality rates in the controls (Figs. 3 and 4) than in the assays where only one high concentration was tested (Fig. 2C and D). This variability we cannot explain, other than to mention that they were done in separate years. Despite the

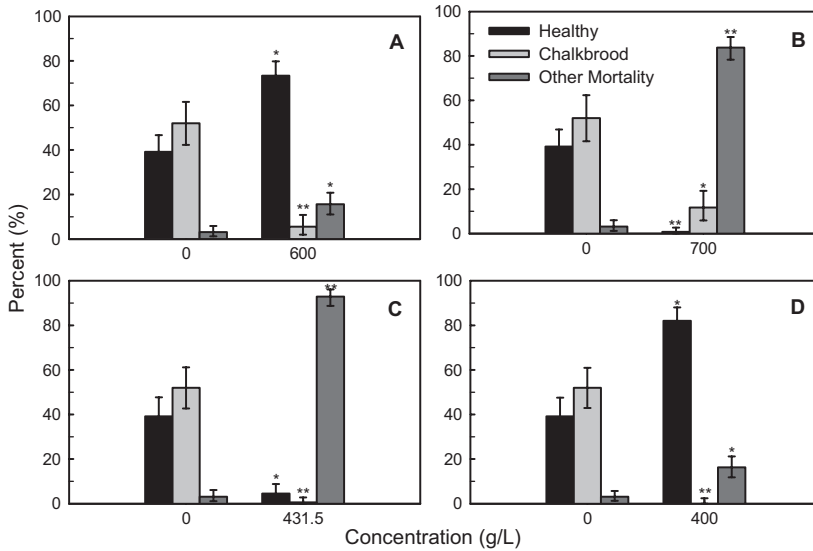


Fig. 2. Effects of (A) Benlate, (B) Captan, (C) Orbit, and (D) Rovral 50 WP on the percentage of *M. rotundata* larvae that completed a cocoon (healthy), died from chalkbrood, or died from other reasons after the provisions were injected with 1  $\mu$ l of fungicide mixture (g [AI]/liter) during the egg stage. Asterisks indicate treatments that are significantly different from controls; \*,  $P < 0.05$  and \*\*,  $P < 0.001$ .

variability in background mortality, the high concentrations of Rovral 50 WP and Rovral 4 F both increased larval mortality  $\approx 13\%$ , while reducing chalkbrood and increasing the number of healthy bees. Orbit, however, seemed to have greater toxicity to the larvae in the first year (Fig. 2C) than in the second year (Fig. 3).

### Discussion

The underlying assumption behind the fungal bioassays is that the less material needed to inhibit fungal growth, the more toxic the fungicide is to *A. aggregata*,

and the greater the potential that fungicide may have in controlling chalkbrood disease in *M. rotundata*. Another reason to favor a more toxic fungicide is that less material will be needed in the field, presumably reducing application costs when used on a commercial scale.

Based on the fungal bioassays alone, Orbit exhibited the best potential for chalkbrood control because it required the lowest concentration to completely inhibit hyphal growth. However, high toxicity to *M. rotundata* eggs and larvae dramatically reduced the potential of Orbit for chalkbrood control. Both Benlate and Rovral 50 WP warrant further study because

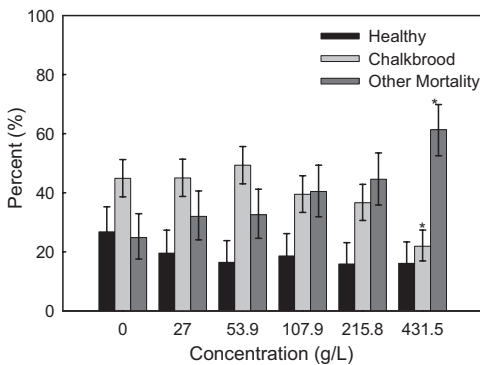


Fig. 3. Effects of Orbit on the percentage of *M. rotundata* larvae that completed a cocoon (healthy), died from chalkbrood, or died for other reasons after provisions were injected with 1  $\mu$ l of fungicide mixture (g [AI]/liter). Treatments were applied when the bees were eggs. Asterisks indicate treatments that are significantly different from controls; \*,  $P < 0.05$ .

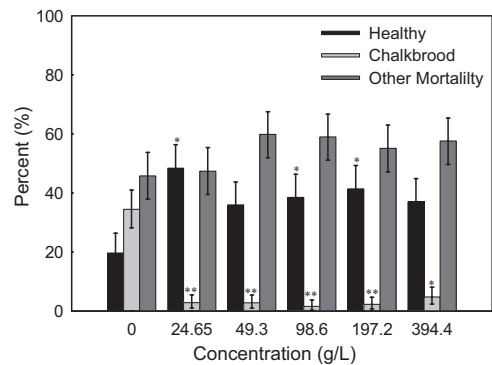


Fig. 4. Effects of Rovral 4 F on the percentage of *M. rotundata* larvae that completed a cocoon (healthy), died from chalkbrood, or died for other reasons after provisions were injected with 1  $\mu$ l of fungicide mixture (g [AI]/liter). Treatments were applied when the bees were eggs. Asterisks indicate treatments that are significantly different from controls; \*,  $P < 0.05$  and \*\*,  $P < 0.001$ .

they were able to completely inhibit fungal growth at modest rates. Captan would be a poor candidate for chalkbrood control due to the large amounts of this fungicide needed to inhibit growth. Complete inhibition of hyphal growth was obtained with Captan, but it may have been achieved, at least in part, by the dilution of nutrients in the medium due to the large amount of Captan added.

In a similar experiment with *A. aggregata*, Goettel et al. (1992) determined that the effective concentration to obtain 50% growth inhibition ( $EC_{50}$ ) for Benlate and Rovral 50 WP was 0.58 and 0.55 mg (AI)/liter, respectively. Our studies determined  $EC_{50}$  values for Benlate and Rovral 50 WP at 3.43 and 8.43 mg (AI)/liter, respectively. The differences in effective concentrations between the two studies may be explained by different strains of *A. aggregata*, but they are more likely due to different culturing conditions. Goettel et al. (1992) grew their cultures on SDAY at 28°C and obtained  $\approx 15$  mm of growth in controls after 14 d. We used a V-8–based medium (James 2004) at 30°C that resulted in 50 mm of growth after 7 d. Cultures grown on SDAY may have already been nutritionally stressed, accounting for smaller  $EC_{50}$  values than those obtained with cultures grown on V-8.

At the higher Rovral 50 WP concentrations tested, no fungal growth occurred on the Rovral 50 WP treatment, except on the initial agar plug that was free of fungicide. By measuring diameter growth from observing the plates from above, the readings for Rovral 50 WP were somewhat exaggerated. Future tests should exclude measuring growth on the initial plug if the growth does not occur on the treated medium. The highest concentrations tested of the other fungicides killed the hyphae on the initial plug.

A bioassay that examines a fungicide's ability to kill *A. aggregata* spores, rather than hyphae, would be a good screening test because spores are the most accessible stage for interrupting the disease cycle; however, such tests are very difficult to conduct because one cannot easily distinguish between actual sporidial effects and compounds that just inhibit germination. Stephen et al. (1982) found a better correlation between fungicide–spore bioassays and chalkbrood incidence in larvae fed fungicides, than with fungicide–hyphal bioassays and chalkbrood incidence, even though they did not distinguish between sporidial effects and inhibition of germination.

The ideal fungicide would considerably reduce incidence of chalkbrood without toxic effects to *M. rotundata* larvae and result in a net increase of healthy bees. Of the fungicides tested, Benlate, Rovral 50 WP, and Rovral 4 F best met these criteria. Although toxicity to the larvae occurred for the highest levels of Benlate, Rovral 50 WP, and Rovral 4 F, this effect was offset with a net increase of healthy bees. Fungicide concentrations required to produce bee toxicity were extremely high and unlikely to be necessary in a field application because reduction of chalkbrood could be obtained at much lower levels. Neither Captan nor Orbit reduced chalkbrood in any of the tests without increasing larval mortality, signifying both fungicides

as toxic to the larvae. Fichter and Stephen (1987) also found that Benlate reduced the incidence of chalkbrood and did not increase bee mortality and that Captan failed to reduce chalkbrood, while increasing larval mortality when these fungicides were incorporated into the artificial pollen provisions.

Atkins and Kellum (1986) fed numerous pesticides to honey bee, *Apis mellifera* L., brood, including benomyl and captan. In their study, benomyl was found to be nontoxic to larvae, whereas captan decreased brood emergence and increased the likelihood of emerged bees having smaller size and deformed wings. In our study, the treated eggs that emerged as adults the following summer had no observable deformities and no measurable mortality occurred between cocoon completion and adult emergence, but we did not measure body size.

We have treated both egg and early instars with fungicides (Huntzinger 2007), but we only report the eggs here because they are more sensitive to fungicides. This result may be due to eggs having a smaller size and greater surface-to-volume ratio than larvae, thus receiving a proportionately higher dose. In addition, the spores in the pollen–nectar provision are exposed to the fungicide for a longer time before being consumed by the larva. Another possibility is that when fungicide treatments occur during the egg stage, the fungicide is present when the larva first begins feeding, compared with treating the provision of a second instar that has already begun consuming pollen and spores, and therefore may already be infected.

We used natural pollen–nectar provisions to rear *M. rotundata* larvae. Other studies have used artificial diets (Goettel et al. 1993, Vandenberg 1994). Advantages of using artificial diets are that a uniform quality and quantity of diet can be fed to each individual, and uniform spore inoculums can be incorporated into the diet. This method also reduces variability among larvae in the timing of fungicide consumption. However, by using the natural pollen provision, we avoided the time-consuming and delicate process of transferring eggs or larvae to artificial provisions. This process can cause mechanical damage and increased mortality (Fichter et al. 1981; our experience). Also, the use of natural provisions should be more relevant to field tests. Goettel et al. (1993) found that bees reared on natural diet were less susceptible to chalkbrood than bees reared on artificial diet. Finally, chalkbrood incidence in our controls was near 50%, indicating sufficiently high levels of spores for the purposes of this study.

Fungicides with promise as chalkbrood control agents also should have low toxicity to *M. rotundata* adults. Ladurner et al. (2005) found that Orbit and Captan were toxic to another megachilid, *Osmia lignaria* Say, when orally administered at high doses in the laboratory. However, nesting female behavior and cell production were not impaired when these products were sprayed on the plants visited by caged *O. lignaria* populations (Ladurner et al. 2008).

Our study demonstrates that the use of certain fungicides can reduce the incidence of chalkbrood dis-

ease and increase the percentage of healthy bees, whereas other fungicides have little or no effect on incidence of chalkbrood disease and increase mortality rates in bee larvae. Benlate, Rovral 50 WP, and Rovral 4 F showed potential for chalkbrood control, and they warrant further study. However, in 2001, DuPont stopped making Benlate (Deer 2001). This detail eliminates Benlate from further consideration and study for a viable chalkbrood control method.

Other species of *Ascosphaera* associated with *M. rotundata* infections, such as *Ascosphaera proliperda* Skou and *Ascosphaera atra* Skou & Hackett (Skou 1972) should be included in bioassay studies with fungicides. Identification of a fungicide that is effective on numerous *Ascosphaera* species might not only contribute to chalkbrood control but also contribute to our understanding of the physiology of *Ascosphaera* fungi. Further studies should include efforts to identify other fungicides that are effective against chalkbrood disease and to develop a practical way to treat chalkbrood in a commercial setting. From our experience with seed growers, they would be willing to treat loose cells or shelters, as long as they did not have to treat repeatedly, as suggested by Parker (1984). James and Pitts-Singer (2005) found adult bees became contaminated with spores when emerging from loose cells, so perhaps treating loose cells would be effective.

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